



Effectiveness of the Combination of Green Betel Leaf Extract (*Piper betle*) and Mint Leaf (*Mentha piperita*) as Antibacterials against *Streptococcus mutans*

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Abstract

The content of betel leaf (*Piper betle*) includes essential oils, alkaloids, flavonoids, phenols, and steroids. Flavonoid compounds in *P. betle* show a mechanism of action in damaging bacterial cell proteins and disrupting irreparable membranes. On the other hand, mint leaves (*Mentha piperita*) contain essential oil ranging from 1-2%, with menthol content around 80-90%, as well as mentone, d-piperitone, hexanol phenyl acetate, ethyl amyl carbinol, and neomenthol. The aim of this study is to determine the effectiveness of the combination of *P. betle* extract and *M. piperita* extract in inhibiting the growth of *Streptococcus mutans* (*S. mutans*) bacteria using the well diffusion method. Extraction of *P. betle* and *M. piperita* extracts were performed using the maceration method. The extraction results showed a yield of 24.94% for the *P. betle* extract and 35.39% for the *M. piperita* extract. The antibacterial testing against *S. mutans* showed an inhibition zone of 21.83 ± 1.58 mm for the single *P. betle* extract (ML), 11.2 ± 1.00 mm for the *M. piperita* (GBL), and 11.76 ± 0.57 mm for the combination extract (GBL/ML). There is an influence of the combination of *P. betle* and *M. piperita* extracts on the antibacterial effectiveness against *S. mutans*, although the inhibition zone of the *P. betle* is larger, measuring 21.83 ± 1.58 mm.

Keywords: Extract, *Streptococcus mutans*, *Piper betle*, *Mentha piperita*

1. INTRODUCTION

Betel leaf (*Piper betle*) contains essential oils, alkaloids, flavonoids, phenols, and steroids [1]. The active components of *P. betle* that act as antiseptics are catechins and tannins, which are polyphenolic compounds [2][3]. The flavonoid compounds found in *P. betle* are believed to work by denaturing bacterial cell proteins and damaging the bacterial cell membrane irreversibly [4]. An activity test of ethyl acetate extract of *P. betle* inhibited the growth of *Staphylococcus epidermidis* [5].

Mentha piperita contains essential oil, menthol, menthone, isomenthone, piperitone, and methyl acetate [6]. The cooling sensation of *M. piperita* provides a comfortable feeling and freshens breath. *M. piperita* extract has antioxidant and antibacterial activities against both gram-positive and gram-negative bacteria [7], *Acne vulgaris* [8], and *S.*

epidermidis [9]. All parts of the *M. piperita* plant can inhibit the growth of pathogenic bacteria in the oral cavity, such as *Streptococcus mutans* [10].

S. mutans is a gram-positive bacterium that plays a key role in dental plaque metabolism [11]. The components of dental plaque from normal microorganisms in the oral cavity can become pathogenic if their population increases, leading to a faster progression of dental caries [12]. *S. mutans* thrives in an acidic environment and can adhere to the tooth surface and each other. The fermentation of their metabolism hydrolyzes sucrose into monosaccharide components, fructose, and glucose. Glucosyltransferase enzymes then assemble glucose into dextran. The residual fructose is the main sugar fermented into lactic acid. The accumulation of bacteria and dextran adheres to the tooth surface, forming dental plaque [13].

In a study conducted by Dinesh et al. [14], the growth inhibitory activity of *S. mutans* bacteria was observed with 20%, 40%, 60%, and 80% concentrations of *P. betle* extract, resulting in inhibition zones of 4.90, 7.20, 10.20, and 12.00 mm, respectively. According to Novita [15], 250 g of *P. betle* yielded 78.2 g (31.28%) of extract after extraction. The obtained extract was tested for its antibacterial activity against *S. mutans*, and it was found that *P. betle* extract inhibited the growth of *S. mutans* bacteria *in vitro*, evidenced by the formation of an inhibition zone around the paper disc

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Table 1. Physicochemical characteristics.

Sample	Moisture (%)	Total Ash Content (%)
<i>P. betle</i>	1.17	0.37
<i>M. piperita</i>	1.08	0.47

measuring 6 mm. The hexane fraction of *P. betle* extract still inhibited the growth of *S. mutans* bacteria at a concentration of 1.25 mg/mL, with an inhibition diameter of 7.20 mm. This concentration is considered the minimum inhibitory concentration (MIC).

M. piperita contains essential oil with inhibitory activity against *S. mutans* bacteria, with a MIC value of 10.5 µg/mL using the diffusion method [6]. Considering the effectiveness of the combination of *P. betle* and *M. piperita* extracts as the antibacterial against *S. mutans*, further research is needed to investigate the effectiveness of this combination. This study could lead to the development of a new antibacterial formulation combining *P. betle* and *M. piperita*, resulting in the discovery of a new antibacterial agent.

2. MATERIALS AND METHODS

2.1. Materials

Streptococcus mutans bacteria were obtained from the Laboratory of the University of Indonesia, while green betel leaf (*P. betle*) and mint leaf (*M. piperita*) were obtained from the collection of the Pharmaceutical Laboratory of STIKES Aisyiyah Palembang.

2.2. Methods

2.2.1. Extract Preparation

Each sample (100 g) was placed in dark glass jars and dissolved in 1 L of 96% ethanol solvent with a ratio of 1:10. This process was repeated three times for 24 h each day, with the material being hand-ground for 15 min. The obtained extract was then filtered using filter paper to obtain a concentrated extract [16].

2.2.2. Physicochemical Analysis

2.2.2.1. Moisture

2 g of sample was placed in an evaporating dish

that had been preheated at 105 °C for 30 min and tared. It was then dried in an oven at 105 °C until a constant weight was reached. After cooling in a desiccator to room temperature, the loss on drying can be calculated using the following equation 1:

$$\text{Moisture (\%)} = \frac{A - B}{A} \times 100\% \quad (1)$$

where A is the weight of the simp before heating (g) and B is the weight of the sample after heating (g).

2.2.2.2. Total Ash Content

The method used in the experiments to determine the mineral content and other inorganic matter in the sample consisted of the desiccation of an amount of 2 g for each sample, in a porcelain crucible. To do so, they were kept in the thermostat at 80 °C for 4 h, after which the samples underwent calcination at 550 °C, in an electric laboratory furnace to constant mass. Total ash content, expressed as the percentage of residue left after dry oxidation by weight (%), was calculated from the following equation, where m_1 is the mass of the porcelain crucible and ash, m_2 is the mass of the empty porcelain crucible, and m_0 is the mass of sample taken.

$$\text{Ash (\%)} = \frac{m_1 - m_2}{m_0} \times 100\% \quad (2)$$

2.2.3. Antibacterial Activity Assay

A modified sterilized cork borer with a diameter of 5 mm was used to create wells in the agar medium. The wells were filled with single extract concentrations, combination extract, positive control, and negative control for testing. Each volume of 40 µL for single *P. betle* extract, single *M. piperita* extract, combination of both extracts, and 20 µL of positive control Ciprofloxacin with a concentration of 20 % in the well, as well as 40 µL of sterile aquadest as the negative control was taken using a micropipette. This process was replicated three times. Placement of the wells on the agar medium had specific requirements, such as each well having the same distance of 2 cm from the edge of the dish, a distance of 3 cm between wells, and a depth of 5 mm. After incubating at 37 °C for 24 h, observation was conducted to determine the presence or absence of inhibition zones around the

wells [17]. The inhibition zones that appear in each perforated agar medium will appear as transparent around the well. They were then measured using a caliper. The diameter of the inhibition zone was measured in mm of the transparent area.

3. RESULTS AND DISCUSSIONS

3.1. Physicochemical Characteristics

Examination of the sample's characteristics includes drying loss and total ash content. The results of the drying loss and total ash content examination can be seen in Table 1. Table 1 shows that the drying loss of *P. betle* and *M. piperita* meets the general requirement, which is less than 10%. The lower the moisture content in the extract, the lower the likelihood of fungal growth in the sample. The purpose of determining the moisture content is to determine the maximum limit or range of water content in the material. This is related to the purity and contaminants in the crude drug. Therefore, reducing the moisture content to a certain level is useful in extending the shelf life of the material during storage.

The determination of total ash content based on the examination in Table 1 provides an overview of the internal and external mineral content that originates from the initial process to the formation of the crude drug. Total ash content is related to both organic and inorganic minerals obtained internally and externally. The function of total ash content is to indicate that the higher the ash content in food material, the lower the quality of the food material.

The yield of the extracts from the maceration process is calculated based on the ratio of the final weight (weight of the obtained extract) to the initial weight multiplied by 100%. Based on the obtained concentrated filtrate compared to the initial extraction mass, the yield of *P. betle* extract is 24.94% and *M. piperita* is 35.39%. The results of the concentrated filtrate can be seen in Table 2.

3.2. Antibacterial Activity

The testing of the antibacterial activity of the individual extract and the combined extract of *P. betle* and *M. piperita* against *S. mutans* was conducted using the well diffusion method with three repetitions. The obtained inhibitory zones are

shown in Table 3. The extract of *P. betle*, with a concentrated extract weight of 5 g, was dissolved in 10 mL of sterile distilled water and tested for its antibacterial effectiveness using the well diffusion method. It has been proven to be highly effective in inhibiting the growth of *S. mutans* bacteria, with a strong inhibitory zone of 21.83 ± 1.58 mm. This is attributed to the presence of essential oil components in *P. betle*, which consist of cavitol, carvacrol, eugenol, estragole, pyrocatechol, and cineole. These components can inhibit the growth of *S. mutans* by inhibiting the activity of the enzyme glucosyltransferase. This enzyme converts sucrose into glucan, which serves as a surface for the attachment of new bacteria to the tooth. Preventing glucan formation creates an environment that is less conducive to the growth of *S. mutans* bacteria [18]-[20]. The essential oil components in *P. betle* also possess antibacterial properties due to the presence of phenol and its derivatives, with cavitol being one of the phenolic derivatives that is five times more potent than phenol itself [21].

The content of cavitol in *P. betle* can denature bacterial cell proteins, thereby impairing the biological activity of the bacteria and rendering the proteins unable to perform their functions. Cavitol works through the same mechanism as phenol but exhibits five times stronger antibacterial effects compared to phenol. As a result, the number of *S. mutans* bacteria on the pellicle is reduced, and the formation of dental plaque is also diminished [22]-[24]. The presence of phenol, which is a toxic compound to bacteria, disrupts the three-dimensional structure of protein without damaging the covalent framework structure. This leads to a change in the properties of the protein. Although the amino acid sequence of the protein remains intact after undergoing such changes, its biological activity is impaired, causing the protein to be unable to perform its function and resulting in cellular dysfunction, lysis, and eventual death.

A small amount of eugenol present in *P. betle* can penetrate and disrupt the fatty acid chains in the

Table 2. Extract yield.

Sample	Mass (g)	Yield (%)
<i>P. betle</i>	100	24.94
<i>M. piperita</i>	100	35.39

Table 3. Antibacterial activity.

Sample	GBL	ML	GBL/ML	CF
Zone Inhibition (mm)	11.20 ± 1.00	21.83 ± 1.58	11.76 ± 0.57	35.83 ± 1.18

Note: GBL : Green betel leaf (*P. betle*); ML : Mint leaf (*M. piperita*) ; CF : Ciprofloxacin

bacterial membrane, leading to increased permeability and subsequent cell membrane damage and death [25]. The essential oil contained in *P. betle* can also reduce the adhesion of *S. mutans* bacteria to the pellicle on the tooth surface. The antibacterial activity of *P. betle* is demonstrated by a decrease in bacterial colony growth, reduced bacterial adhesion ability, and a decrease in the activity of the enzyme glucosyltransferase (GTF) produced by the bacteria. Consequently, bacterial growth is inhibited, leading to a reduction in dental plaque formation [26].

Based on the results in Table 3, the antibacterial test was conducted by dissolving 5 g of concentrated extract in 10 mL of distilled water in a flask. It was found that *M. piperita* extract has proven antibacterial effectiveness with a strong inhibitory zone of 11.2 ± 1.00 mm. According to Oyedele and Afolayan [27], the essential oil of *M. piperita* contains compounds such as menthol, which belongs to the terpenoid group of monoterpenes and exhibits antimicrobial activity. Flavonoids in mint leaves function as antibacterials by forming extracellular protein complexes that disrupt the integrity of bacterial cell membranes, while tannins have antibacterial properties by causing bacterial cell walls or membranes to contract, thereby disrupting bacterial permeability.

This can result in bacterial cells being unable to perform vital activities, leading to inhibited growth [28].

P. betle contains carotene, thiamine, riboflavin, niacin, vitamin C, tannins, glucose, starch, and amino acids [29]. *M. piperita* contains antioxidant compounds such as flavonoids, phenolic acids, triterpenes, vitamin C, provitamin A, minerals, phosphorus, iron, calcium, and potassium [30]. When *P. betle* and *M. piperita* are combined as an antibacterial treatment for *S. mutans*, their effectiveness is lower compared to the single extract of *P. betle*. This is because *P. betle* contains vitamin C, niacin, and amino acids, while *M. piperita* contains phenolic acids and vitamin C. When these compounds are combined, the acidic properties increase in the extract. The production of acid lowers the pH within minutes, resulting in increased growth of *S. mutans* and reduced antibacterial activity. *S. mutans* is acidogenic, capable of producing a pH < 5 within 1-3 min compared to other bacteria [31]. Acidic environments can enhance the growth of acid-tolerant bacteria like *S. mutans* [32]. This bacterium also has the ability to synthesize sucrose, glucose, or other carbohydrates into extracellular polysaccharides and acids [33]. In the research conducted, the combined extract of *P. betle* and *M. piperita* exhibited strong antibacterial effectiveness against *S. mutans* with an inhibitory zone of 11.76 ± 0.57 mm (Figure 1). The extract used was 5 g of *P. betle* extract and 5 g of *M. piperita* extract, dissolved in 10 mL of distilled water.

Ciprofloxacin was used as a positive control in the study and had the largest inhibitory zone of 35.83 ± 1.18 mm. Ciprofloxacin is used for the treatment of infections caused by Gram-negative bacteria such as *E. coli*, *P. mirabilis*, *Klebsiella sp*, *Shigella sp*, *Enterobacter sp*, *Haemophilus sp*, *Chlamydia sp*, *Salmonella sp*, *P. aeruginosa*, as well as certain Gram-positive bacteria such as *Staphylococcus sp* and *Streptococcus sp* [34]. Incidentally, it may cause side effects such as

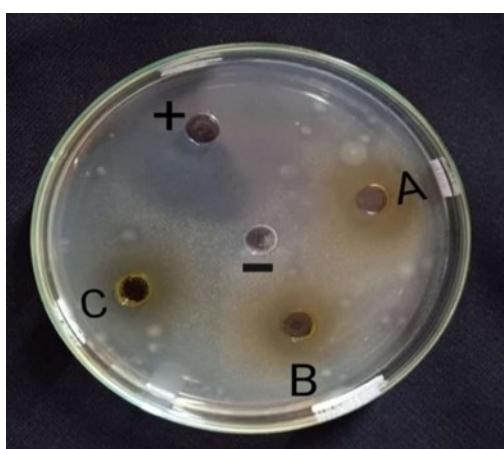


Figure 1. Zone inhibition A) *M. piperita*; B) *M. piperita/P. betle*; C) *P. betle*; +) Ciprofloxacin; -) Aquadest.

crystalluria or hematuria [35]. The mechanism of action of the antibiotic Ciprofloxacin involves inhibiting nucleic acid synthesis. This group of antibiotics can enter the cell through passive diffusion via water-filled protein channels (porins) on the outer membrane of bacteria, and they uniquely disrupt the function of DNA gyrase (topoisomerase II) during bacterial growth and reproduction, thus inhibiting bacterial DNA replication [36]. Mechanisms of Ciprofloxacin resistance happen through Fluoroquinolone antibiotics, including ciprofloxacin, which bind to the β subunit of the DNA gyrase enzyme and block the activity of this essential enzyme in maintaining DNA supercoiling, which is important for DNA replication. Mutations in the genes encoding DNA gyrase can lead to the production of an active enzyme that is unable to bind to fluoroquinolones [37].

4. CONCLUSIONS

The combination extract of green betel leaf (*P. betle*) and mint leaf (*M. piperita*) has antibacterial effectiveness against *Streptococcus mutans*, as evidenced by the inhibitory zone of 11.76 ± 0.57 mm. In comparison, the widest inhibitory zone was observed with the single extract of *P. betle*, measuring 21.83 ± 1.58 mm. The inhibitory zone formed by the single extract of *M. piperita* was 11.2 ± 1.00 mm. However, the combined extract of them had lower antibacterial effectiveness against *S. mutans* compared to the single extract of *P. betle*.

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Author Contributions

I. K. investigation, methodology; M. T. investigation, data curation; A. F. conceptualization, methodology, writing original draft preparation.

Conflicts of Interest

The authors declare no conflict of interest.

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